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Satellite tobacco ringspot virus RNA: A subset of the RNA sequence is sufficient for autolytic processing

(plant virus/*in vitro* transcription/RNA processing efficiency)

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ABSTRACT **The satellite RNA of tobacco ringspot virus depends upon tobacco ringspot virus for its replication and source of coat protein. The satellite RNA reduces virus accumulation and the severity of virus-induced symptoms. Repetitive sequence, dimeric, and higher forms of the satellite RNA are known to autolytically process to form biologically active monomeric RNA of 359 nucleotide residues [Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R. & Bruening, G. (1986) *Science* 231, 1577–1580], with a 5′-hydroxyl and a 2′,3′-cyclic phosphodiester as the new terminal groups. We show here that transcripts of full-length and truncated DNA clones of the satellite RNA sequence also process in a nonenzymic reaction. One such transcript was an RNA that has about one-fourth of the satellite RNA sequence, representing the 3′-terminal and 5′-terminal portions of monomeric RNA joined in the junction that is cleaved in dimeric RNA. This RNA autolytically processed more efficiently than molecules with a larger proportion of the satellite RNA nucleotide sequence.**

The satellite RNA of tobacco ringspot virus (STobRV RNA) (1) is one of several small satellite RNAs of plant viruses (2–4). A satellite RNA replicates only in cells that are infected by its supporting virus, whereas the supporting virus replicates in its hosts regardless of whether the satellite RNA is present. STobRV RNA acts as a parasite of any of several strains of tobacco ringspot virus (TobRV). It is encapsidated in TobRV coat protein and in this stable form is readily transmitted with its supporting virus. The 359-nucleotide residue sequence (5) of STobRV RNA occurs in virus particles not only in this linear "monomeric" form but also as linear repetitive sequence dimers, trimers, and higher-order multimers (6).

The STobRV RNA nucleotide sequence is responsible for at least four functions. They are (*i*) recognition as template by RNA replication machinery of TobRV or the host plant or some combination thereof, (*ii*) encapsidation by TobRV coat protein, (*iii*) reduction in TobRV yield and in the severity of symptoms induced by TobRV alone, and (*iv*) the recently discovered (7) autolytic processing of dimeric and trimeric forms of the satellite RNA at the junction between monomeric STobRV RNA units. This reaction produces biologically active, monomeric STobRV RNA with new termini of a 5′-hydroxyl group and a 2′,3′-phosphodiester. Autolytic processing may have a role in STobRV RNA replication (8).

Our presumption is that no one of these four functions will require, individually, the entire STobRV RNA sequence (7). Linear RNAs were synthesized that have circularly permuted STobRV RNA sequences, so that 3′-terminal and 5′-terminal STobRV RNA sequences are joined in a phosphodiester bond that corresponds to the junction between monomeric units in multimeric STobRV RNA. We tested various truncated forms of these RNAs for their ability to undergo autolytic processing.

MATERIALS AND METHODS

Plasmids. Plasmid pSP651 (9) has a circularly permuted dimeric copy of the STobRV RNA sequence inserted at the restriction endonuclease *Bam*HI site of pSP65, a plasmid that bears a bacteriophage SP6 promoter (10). The insert begins at residue 244 of the monomeric STobRV RNA sequence and ends at residue 243, both of which are in the single *Sau*3AI site of the sequence (5). Taking into account the coincidence of vector and satellite RNA sequences, the construction has STobRV RNA sequences that begin at residue 243 and end at residue 247.

Plasmid pSP65T has a circularly permuted monomeric STobRV RNA sequence that begins at residue 278 and ends at residue 277. It was constructed by ligating pSP65 that had been linearized with *Sma* I and the circularly permuted monomeric STobRV RNA sequence derived from *Taq* I digestion of a precursor of pSP651, pBB1 (9). Before ligation, the latter was incubated with Klenow fragment DNA polymerase, dCTP, and dGTP to produce flush ends (ref. 11, pp. 113–114).

The insert for plasmid pSP65H13 is the small junction-containing *Hae* III fragment (5) from the pSP651-derived plasmid pSP653 (9). The vector for this construction was *Sma* I-linearized pSP65. pSP65H13 has residues 321–359 of STobRV RNA on the 5′ side of the junction and 1–58 on the 3′ side.

***In Vitro* RNA Synthesis.** Ten micrograms of plasmid was linearized by treatment with the restriction endonuclease indicated. The DNA, recovered after extraction with phenol/chloroform and precipitation by ethanol, was incubated (10) at 40°C for 1 hr in 200 μl of 40 mM Tris·HCl, pH 7.5/20 mM NaCl/6 mM $MgCl_2$/2 mM spermidine hydrochloride/10 mM dithiothreitol containing 250 μM each rNTP, including 150 μCi (1 Ci = 37 GBq) of [α-^{32}P]GTP or [γ-^{32}P]GTP, 200 units of the ribonuclease inhibitor RNasin (Promega Biotec, Madison, WI), and 200 units of bacteriophage SP6 RNA polymerase (New England Biolabs). After extractions with 200 μl of phenol/chloroform and subsequent precipitation, nucleic acids were resolved by electrophoresis through polyacrylamide gel in TBE buffer (ref. 11, pp. 156, 174–178) and 7 M urea.

RESULTS

The authenticity of the insert in pSP651 is shown by the previously reported (9) autolytic and biological activities of

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Abbreviations: TobRV, tobacco ringspot virus; STobRV RNA, satellite tobacco ringspot virus RNA.

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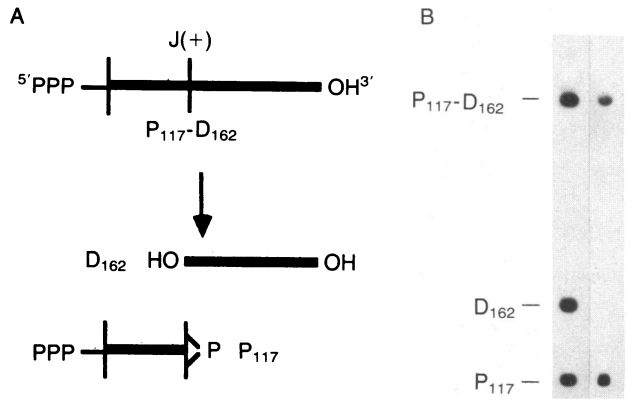

FIG. 1. Autolytic processing of a three-quarter-length segment of circularly permuted STobRV RNA. (*A*) Diagram of the *in vitro* primary transcript (P_{117}-D_{162}) from *Alu* I-digested plasmid pSP651 (9) and the expected products of its autolytic processing. J(+) locates the CpA phosphodiester bond between residues 359 and 1. This bond is cleaved during autolysis of multimeric STobRV RNA that is of the polarity of encapsidated RNA, defined as (+) polarity (7). The products are the promoter-proximal fragment (P_{117}) and the promoter-distal fragment (D_{162}). The subscripts indicate the numbers of nucleotide residues of each RNA that correspond to STobRV RNA sequences. The P_{117} RNA has a triphosphate-terminated chain of an additional 19 nucleotide residues derived from the vector, as indicated by the thin lines. The 3'-hydroxyl, 5'-hydroxyl, 2',3'-cyclic phosphodiester, and 5'-triphosphate terminal groups are marked. (*B*) Plasmid pSP651 was completely digested with restriction endonuclease *Alu* I, and the DNA fragments were incubated with bacteriophage SP6 RNA polymerase and either [α-^{32}P]GTP to label all RNAs (left lane) or [γ-^{32}P]GTP to label RNAs that contain the P_{117} sequence (right lane). The transcripts, after electrophoresis through a 6.5% polyacrylamide gel, were detected by autoradiography. All three zones were detected in both lanes by staining (12) with toluidine blue 0 (not shown).

transcripts of the *Bam*HI-linearized plasmid. A circularly permuted dimeric sequence necessarily contains one unpermuted copy of the monomeric RNA sequence bounded by two junction sites that join it to the two bordering, fragmentary STobRV RNA sequences. We represent the primary transcript nucleotide sequence in 5'-to-3' orientation as P-M-D, where P and D are the promoter proximal and promoter distal bordering sequences, respectively, and M is the nucleotide sequence of monomeric STobRV RNA.

We found that reaction mixtures contained the anticipated transcript P-M-D. However, autolysis during transcription is expected because it is facilitated by the Mg^{2+} and spermidine that are components of the polymerase reaction mixture (7). Indeed, the *in vitro* transcript autolytically processed at both junction sites—i.e., at two specific CpA phosphodiester bonds (7, 5)—to release monomeric RNA, M, and the two bordering RNA fragments, P and D. These three RNAs were recognized by their electrophoretic mobilities, by the identities and phosphorylation state of their 5'-terminal residues, by the reduced mobility of D and D-containing RNAs when the plasmid was linearized at a site more distant from the promoter, and by partial sequence analysis (methods in ref. 5). Initiation of transcription by SP6 RNA polymerase within the insert of pSP651 is *not* a reasonable explanation for the less-than-dimer-sized RNAs that were observed, because pSP651 that was linearized at a site between the promoter and insert, by *Eco*RI, failed to give transcripts that could be detected after gel electrophoresis (data not shown).

Of the two junction sites in pSP651 the one that is closest to the SP6 promoter, the P-M junction, is most amenable to truncation of the adjacent nucleotide sequences. pSP651 was digested with restriction endonuclease *Alu* I, which cuts within the M sequence such that 162 nucleotide residues remain between the junction site and the 3' end of the DNA fragment. The pSP651 construction has 19 nucleotides from the vector and 117 residues from STobRV RNA between the transcription start site and the junction. Thus we designate the primary transcript of *Alu* I-digested pSP651 as P_{117}-D_{162}. P_{117}-D_{162}, which contains 77% of the circularly permuted STobRV RNA sequence, autolytically processed to RNA fragments P_{117} and D_{162} (Fig. 1). Identities of the three RNA zones in Fig. 1 were assigned according to expected mobilities. Determination of the 5'-terminal groups and partial nucleotide sequence analyses, as described (5), confirmed

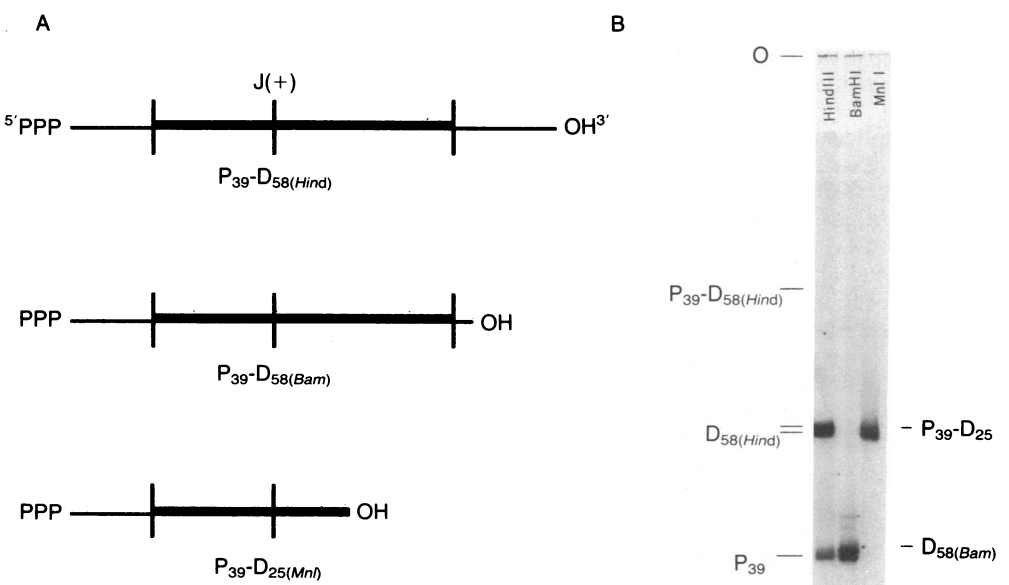

FIG. 2. Autolytic processing of a transcript of a small junction-site-containing cDNA clone of the STobRV RNA sequence. The template was plasmid pSP65H13, which has as its insert the small *Hae* III fragment (5) of cloned STobRV RNA cDNA (9). (*A*) Diagram of the expected primary transcripts after template is cut with *Hin*dIII, *Bam*HI, and *Mnl* I in separate experiments. Transcripts have 25 nucleotide residues to the 5' side of the STobRV RNA insert, and $D_{58(Bam)}$ and $D_{58(Hind)}$ are expected to have 3 and 30 vector nucleotide residues, beyond the STobRV RNA sequences, at their respective 3' ends. (*B*) Analyses of the products of transcription reactions. Transcription reaction mixtures were as in Fig. 1 except that RNAs were resolved by electrophoresis through a 12% polyacrylamide gel. Symbols are as in Fig. 1. O, origin.

the identities of P_{117} and D_{162}. Since P_{117}, D_{162}, and P_{117}-D_{162} were the only RNAs observed in significant amounts, the sequence does not appear to have termination signals that SP6 RNA polymerase recognizes. Weak termination was observed during the transcription of *Bam*HI-linearized pSP651 (data not shown), but the termination site is not within the nucleotide sequence of P_{117}-D_{162}.

The construction of plasmid pSP65T places the junction site 28 residues closer to the SP6 promoter than it is in pSP651, considering both vector and STobRV RNA sequences. Digestion of pSP65T with *Alu* I released a fragment that should be transcribed into P_{82}-D_{162}. This transcript processed in a fashion similar to that of P_{117}-D_{162} in Fig. 1.

To test whether processing occurs in the absence of transcription, the primary transcripts P_{117}-D_{162} and P_{82}-D_{162} were electrophoretically purified under denaturing conditions and were separately incubated at room temperature or at 40°C for 30 min in 200 μl of 40 mM Tris·HCl, pH 7.5/20 mM NaCl/6 mM $MgCl_2$/2 mM spermidine hydrochloride. The intact two-junction transcript of pSP651 and its two single-junction partial autolysis products also were purified and incubated under these conditions. Autolysis occurred at each of the junction sites in these molecules (data not shown) just as it does in dimeric (one-junction) and trimeric (two-junction) STobRV RNA from virus particles (7).

Plasmid pSP65H13 contains a sequence of 97 nucleotide residues derived from STobRV RNA. pSP65H13 was digested in separate reactions with *Hin*dIII, *Bam*HI, and *Mnl* I, the last enzyme cutting within STobRV RNA sequences (Fig. 2). The results in Fig. 2*B* show that both primary transcripts P_{39}-$D_{58(Hind)}$ and P_{39}-$D_{58(Bam)}$ processed very efficiently, the former primary transcript surviving in at most a trace amount (Fig. 2*B*, left lane) and the latter not detectable (center lane). The identities of the RNA zones, as indicated beside the autoradiogram in Fig. 2, were postulated from their mobilities and were confirmed by partial nucleotide sequence determination of 5′-end-labeled RNAs. The $D_{58(Hind)}$ fragment consistently was observed to be a doublet, presumably because of variable run-off termination. Digestion of pSP65H13 with *Mnl* I and subsequent transcription with SP6 RNA polymerase produced the primary transcript P_{39}-D_{25}. This RNA produced no detectable autolysis product (Fig. 2*B*, right lane). That is, P_{39}-D_{58} exhibited autolytic processing, but P_{39}-D_{25} did not.

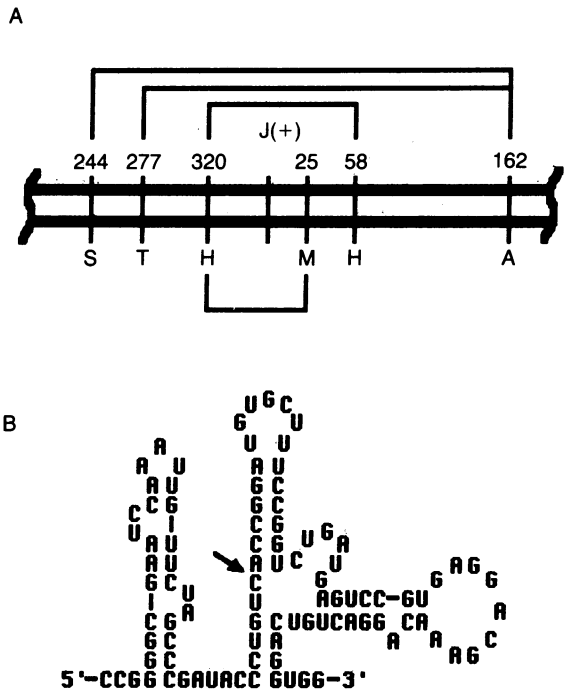

FIG. 3. Summary of the autolytic processing of *in vitro* (+) polarity transcripts of cloned truncated STobRV RNA sequences. The constructions were derived from clones of circularly permuted monomeric STobRV RNA cDNA. (*A*) Diagram representing an almost complete circularly permuted STobRV RNA sequence. Transcripts that exhibited autolytic processing are indicated by the brackets above the diagram, whereas the transcript that failed to process is indicated below the diagram. Selected restriction endonuclease sites are represented by letters below the diagram: S, *Sau*3AI; T, *Taq* I; H, *Hae* III; M, *Mnl* I; A, *Alu* I. Numbers designate the nucleotide residue that is to the 5′ side of the restriction endonuclease cutting site. (*B*) Nucleotide sequence of the smallest portion of the STobRV (+)RNA sequence that was shown to undergo autolytic processing in the experiments reported here, corresponding to the small *Hae* III fragment of STobRV RNA cDNA. The secondary structure shown is of no known significance. It was developed by taking the three stems calculated (13) to be most stable and excluding other, incompatible, stems. The arrow marks the autolytically cleaved phosphodiester bond.

DISCUSSION

The results from the analyses of transcripts of the various plasmid constructions are summarized in Fig. 3*A*. The top bracket of Fig. 3*A* shows the results from transcription of an *Alu* I-cut plasmid that has a *Sau*3AI-to-*Sau*3AI insert; the second bracket shows the results from transcription of an *Alu* I-cut plasmid that has a *Taq* I-to-*Taq* I insert. The transcripts of plasmid pSP65H13 (third bracket and bottom bracket of diagram) are described in Fig. 2. As indicated by the diagram, when 64 nucleotide residues (39 of which are from satellite sequence) were present on the 5′ side of the junction, the presence on the 3′ side of more than 25 nucleotide residues from STobRV RNA sequences, but possibly fewer than 64 (58 of which are satellite sequence), was sufficient for the autolytic reaction.

Thus no more than 97 nucleotide residues of STobRV RNA origin were needed for the self-processing reaction. The short 5′- or 3′-terminal nonsatellite sequences that were derived from plasmid sequences did not interfere with autolytic processing. A construction that is similar to pSP65H13, but with a bacteriophage T7 promoter, gave similar results after linearization of the plasmid and transcription. Since autolysis has been observed for RNA that was synthesized in TobRV-infected plants (7) and for transcripts synthesized *in vitro* by two different RNA polymerases, it is unlikely that these autolysis reactions have any factor in common other than spermidine and Mg^{2+} ions. Thus our analyses of the purified junction-containing RNAs show that no plant enzyme and no other protein of any kind is required for autolytic processing.

The autolytic reaction that was observed for dimeric STobRV RNA from virus particles (7) also is a property of a "core" contiguous STobRV RNA sequence that flanks the junction (Fig. 3*B*). The dimeric STobRV RNA from virus particles (7), the circularly permuted dimeric RNA sequence transcribed from pSP651, and the sequences that represent a substantial fraction of the monomeric STobRV RNA sequence, as in Fig. 1, all autolytically processed to a more limited extent than did P_{39}-D_{58}. Apparently, additional STobRV RNA sequences, when linked to this core sequence, reduced the ability of the core to perform the autolytic reaction. It is possible that modulation of autolytic processing of the core sequence is a biologically significant control function of STobRV RNA sequences that lie outside the core.

The new termini generated in self-cleavage reactions of STobRV RNA and certain other small RNAs (14, 15) bear a 5′-hydroxyl group and a 2′,3′-phosphodiester group. Thus autolytic cleavage of STobRV RNA may be an example of a particularly primitive RNA reaction: the new termini are those formed during ordinary base- or acid-catalyzed hydrol-

ysis of RNA, and the only cofactor required is a structure-stabilizing ion such as magnesium. In contrast, the intermediates in RNA self-splicing (16) and in catalytic RNA reactions (17) have 5′-phosphate and 2′,3′-vicinal hydroxyl groups at the new termini, and the cofactor requirements are more complex and specific.

The small size of the autolytically processing RNA core that we have identified makes it particularly suitable for mutagenesis experiments aimed at identifying functional and structural relationships between nucleotide residues in the core.

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